ORIGINAL ARTICLE 10.1111/j.1469-0691.2004.00876.x

Diversity of *Listeria monocytogenes* isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis

S. Lukinmaa[1], *K. Aarnisalo*[2], *M.-L. Suihko*[2] *and A. Siitonen*[1]

[1]Laboratory of Enteric Pathogens, National Public Health Institute, Helsinki and [2]VTT Biotechology, Espoo, Finland

ABSTRACT

Automated ribotyping, pulsed-field gel electrophoresis (PFGE) and serotyping were evaluated for the epidemiological study of isolates of *Listeria monocytogenes* collected in Finland in 1997–1999 from human blood ($n = 116$) and the food industry ($n = 72$). The isolates divided into six serotypes, 23 *Eco*RI ribotypes, 54 *Asc*I PFGE types, and 57 final subtypes if all results were combined. The discrimination index of ribotyping was lower (0.873) than that of PFGE (0.946). Two final subtypes dominated among human isolates, and identical subtypes were also found among food industry isolates. All PFGE types were serotype-specific, whereas two ribotypes included isolates of two serotypes. Isolates of serotype 3a, involved in an outbreak in Finland in 1999, matched one of these ribotypes, which also included some food industry isolates of serotype 1/2a. Ribotyping with *Eco*RI would not have been sufficient to define the outbreak in Finland caused by serotype 3a isolates. Although ribotyping is applicable as the first method in outbreak situations, human and food isolates with identical ribotypes should be investigated further by PFGE.

Keywords Epidemiology, *Listeria monocytogenes*, pulsed-field gel electrophoresis, ribotyping, serotyping, typing

Original Submission: 17 December 2002; **Revised Submission:** 28 March 2003; **Accepted:** 21 April 2003

Clin Microbiol Infect 2004; 10: 562–568

INTRODUCTION

Listeria monocytogenes is an important food-borne pathogen that causes listeriosis in humans. It is found commonly in soil, in water and on plant material. Its ability to survive for long periods under adverse environmental conditions, and to colonise, multiply and persist on processing equipment, makes it a particular threat in the food industry.

During the past 20 years, several outbreaks of listeriosis have been described. Various food items, e.g., coleslaw in Canada [1], paté in the UK [2], soft cheese in Switzerland [3], rillettes in France [4], rice salad [5] and sweet corn in Italy [6], pasteurised milk [7], Mexican-style cheese [8], shrimp [9], chocolate milk [10], hot dogs and deli meats [11] in the USA, and butter in Finland [12], have been implicated. Most of these outbreaks involved invasive infections caused by isolates of serotype 4b [1–4,7–9,11] and 3a [12], but two involved gastrointestinal non-invasive infections with fever caused by serotypes 1/2b [5,10] and 4b [6]. In Finland, listeriosis has been connected only on three occasions to a specific food source, namely salted mushrooms in 1989 [13], rainbow trout in 1998 [14], and butter in 1999 [12]. The serotypes of the causative isolates found in these foodstuffs were 4b, 1/2a and 3a, respectively.

Serotyping is a classic epidemiology tool [7,15]. However, when compared with other subtyping methods, it has poor discriminatory power [16]. Thirteen serotypes of *L. monocytogenes* are recognised, but most of the isolates that are important in public health have been classified as serotypes 1/2a, 1/2b or 4b [17,18]. Other reported typing methods include phage-typing [19], multilocus enzyme electrophoresis [20], ribotyping [21], pulsed-field gel electrophoresis (PFGE) [22],

Corresponding author and reprint requests: A. Siitonen, National Public Health Institute, Laboratory of Enteric Pathogens, Mannerheimintie 166, FIN-00300 Helsinki, Finland
E-mail: anja.siitonen@ktl.fi

amplified-fragment length polymorphism analysis [23] and randomly amplified polymorphic DNA analysis [23]. PFGE is one of the most discriminating and reproducible methods for the subtyping of *L. monocytogenes* [22,24], but is time- and labour-intensive. Thus, automated ribotyping [25], which is also very reproducible, may offer several benefits over manual molecular methods [26], although the suitability of automated ribotyping for epidemiological studies of *L. monocytogenes* has not been clarified. Hollis *et al.* [27] have made this type of comparison, but no *Listeria* isolates were included in their study.

In Finland, ribotyping has not been used previously to study the epidemiology of *L. monocytogenes* isolates of human origin. The purpose of this study was to evaluate the usefulness of automated ribotyping and PFGE, in combination with serotyping, in differentiating between isolates for the detection of infection clusters and their sources, as well as in tracing sporadic cases of listeriosis. These methods were applied to subtype *L. monocytogenes* isolates from human blood and food industry specimens, collected during the same period in Finland.

MATERIALS AND METHODS

Isolates

Isolates of *L. monocytogenes* were collected in Finland in 1997–1999 from human ($n = 116$) and food industry ($n = 72$) samples (Table 1). All human isolates (one/patient) were isolated from blood cultures by standard methods in clinical microbiology laboratories, and were submitted subsequently to the Laboratory of Enteric Pathogens at the National Public Health Institute (Helsinki, Finland) for verification and serotyping. Identification of the strains was by standard methods [28,29]. Twenty-five human isolates were connected with a listeriosis outbreak [12]. The food industry isolates were collected during hygiene surveys in 11 food-processing plants: one meat, one poultry, one ready-to-eat food, three dairy and five fish plants. The samples were taken from equipment in contact with the food being processed, from surfaces not in direct contact with the product, such as tables, floors, doors, drains, tools and aprons, as well as from raw materials and products. The enrichment, cultivation and preliminary identification method used was that recommended by the Nordic Committee on Food Analysis [30]. For ribotyping (see below), the identification databases of DuPont Qualicon (Wilmington, DE, USA) and VTT (Espoo, Finland) were also used to confirm the identification of the isolates. Isolates were stored at −70°C in sterilised skimmed milk (human isolates) or in glycerol 5% v/v (food isolates).

Table 1. Source of *Listeria monocytogenes* isolates included in the study

Source	Number of isolates ($n = 188$)		
	1997 ($n = 53$)	1998 ($n = 80$)	1999 ($n = 55$)
Human ($n = 116$)			
Blood	41	35	40
Food industry ($n = 72$)			
Fish	12	12	4
Dairy	0	2	5
Meat	0	14	0
Ready-to-eat foods	0	0	6
Poultry	0	17	0

Serotyping

Isolates were serotyped using antisera against somatic (O) and flagellar (H) antigens according to the instructions of the manufacturer (Denka Seiken, Tokyo, Japan) with minor modifications. Briefly, isolates were revived by growth on sheep blood agar (Oxoid, Basingstoke, UK) overnight at 37°C before inoculation on brain–heart infusion (BHI) agar (Difco, Detroit, MI, USA). A bacterial suspension in NaCl 0.2% w/v was then heated for 1 h at 100°C for determination of the O antigens. For determination of H antigens, isolates were passed four times at 25°C through semi-liquid BHI broth in Craigie's tubes (containing agar 0.2% w/v).

Ribotyping

The isolates were ribotyped using the automated RiboPrinter System (Dupont Qualicon) following the manufacturer's standard instructions, as described by Bruce [31]. Bacterial cells were picked from sheep blood agar (human isolates) or BHI agar (food isolates). DNA was digested with *Eco*RI (DuPont Qualicon). The automated system processed the batches and generated a pattern for each sample and marker lane using proprietary algorithms. Isolates were assigned a ribogroup from the data base, or a new one was created, and similarities were calculated (Qualicon software v. 12.2). A ribogroup was defined as a set of closely related patterns (threshold similarity ≥0.96) that were mathematically indistinguishable from one another by the system [31]. The ribogroup patterns were composite patterns for all the isolates of the group, analysed with the same instrument. The isolates were called ribotypes and had the same code as the relevant ribogroup, i.e., RT-1–RT-23. The patterns of all new ribogroups were analysed three times to test their reproducibility.

PFGE

DNA preparation from *L. monocytogenes* isolates was performed as described previously [32]. The isolates were grown on blood agar overnight at 37°C, and then for 17–18 h at 37°C in BHI broth. The BHI broth culture (2 mL) was mixed with 5 mL of cold PIV buffer (10 mM Tris pH 7.5, 1 M NaCl) and centrifuged at 3000 *g* for 15 min at 4°C. The pellet was resuspended in 750 μL of cold PIV buffer and mixed in equal parts with molten low-melting-point agarose (Sea Plaque agarose; FMC BioProducts, Rockland, ME, USA) 2% w/v. The mixture was pipetted into plug moulds. The plugs were incubated overnight at 37°C in EC buffer (6 mM Tris-HCl

pH 7.5, 1 M NaCl, 100 mM EDTA, Brij-58 0.5% w/v, sodium deoxycholate 0.2% w/v, sodium lauroylsarcosine 0.5% w/v) with lysozyme 1 mg/mL. The plugs were then incubated overnight at 55–57°C in ES buffer (0.5 M EDTA pH 9.5, sodium lauroylsarcosine 1% w/v) with proteinase K 0.3 mg/mL. Washing of the plugs and conditions for restriction endonuclease digestion and PFGE were as described previously [33]. Chromosomal DNA was digested overnight with 5 U of *Asc*I (New England BioLabs, Beverly, MA, USA). Electrophoresis was performed at 210 V on agarose (Pronadisa D-5; Hispanlab, Madrid, Spain) 1% w/v gels with the CHEF Mapper or CHEF-DR systems (Bio-Rad Laboratories, Richmond, CA, USA). Running conditions for *Asc*I-digested DNA comprised pulse times of 1–28 s for 10 h, followed by 28–30 s for 10 h. Low Range PFG Markers (New England BioLabs) were used as molecular size standards. The gels were photographed with an AlphaImager 1220 (Alpha Innotec Corporation, San Leandro, CA, USA). The images were analysed by BioNumerics software (Applied Maths, Kortrijk, Belgium) and normalised by means of the size standards on each gel. Banding patterns from each normalised image were compared with the *L. monocytogenes* PFGE types of human isolates collected since 1990. Any difference between two profiles was considered sufficient to distinguish two different PFGE profiles. The new PFGE profiles were marked by successive numbers, including a letter V if the profile of a food industry isolate had not been found previously in the collection of *L. monocytogenes* isolates of human origin. Similarity values were calculated by the unweighted pair-group method with arithmetic averages and the DICE coefficient by the BioNumerics software.

The discriminatory power

The discriminatory power of each typing method was determined by calculating the discriminatory index (DI) [34].

RESULTS

Serotyping (Table 2) divided the 188 isolates into six O:H serotypes: serotype 1/2a (59 human, 57 food industry isolates); 1/2b (one human isolate); 1/2c (five food isolates); 3a (29 human and four food isolates); 3b (one food isolate); and 4b (27 human and five food isolates). The DI for serotyping was 0.562. Table 3 summarises the number of ribotypes and PFGE types contained in each serotype.

Automated ribotyping divided the isolates into 23 *Eco*RI ribotypes (Table 2). Eight of the ribotypes were generated only from human isolates and were new to the data base, seven included both human and food isolates, and eight comprised food industry isolates only. There were three main ribotypes (RT-1, RT-2, RT-4), which were related closely to each other (similarity 0.90–0.94), and contained 76 (66%) of the 116 human isolates and 33 (46%) of the 72 food industry isolates. The DI for ribotyping was 0.873, while the DI values for the most common serotypes 1/2a, 3a and 4b were 0.803, 0.229 and 0.762, respectively. Table 4 summarises the number of serotypes and PFGE types in each ribotype.

Enzyme *Asc*I divided the isolates into 54 PFGE types (Table 2). Eleven of these types included both human and food isolates. The three main PFGE types were 1, 5 and 71. The DI for PFGE was 0.946. All PFGE patterns were serotype-specific; i.e., identical PFGE types were not found among different serotypes. Three PFGE types (27, 42 and V19) were each divided into two different ribotypes: RT-6 and RT-14 (similarity 0.92), RT-3 and RT-4 (similarity 0.92), and RT-8 and RT-13 (similarity 0.86), respectively. Two of the ribotypes (RT-1 and RT-4) contained isolates of two serotypes (1/2a and 1/2c, and 1/2a and 3a, respectively). The three main ribotypes (RT-1, RT-2, RT-4) contained 11, seven and four PFGE types, respectively (Table 4). Similarity values of PFGE types among the isolates of ribotype RT-1, RT-2 and RT-4 were *c.* 50–93%, 75–100% and 70–87%, respectively (data not shown).

When the results of serotyping, ribotyping and PFGE were combined, 57 final subtypes were obtained (Table 2). Ten of these subtypes contained both human and food isolates, 21 were found only among isolates from the food industry, and 26 only among isolates from humans. Three of the 57 final subtypes (1, 9 and 42) contained 36% of the isolates (51% of the human isolates and 11% of the food isolates).

DISCUSSION

Isolates of *L. monocytogenes* from patients with invasive listeriosis and from the food-processing industry were analysed by serotyping, automated ribotyping and PFGE. All 188 isolates were typeable by the three methods used. The discriminatory power of ribotyping and PFGE depends on the restriction enzymes used, and *Eco*RI and *Asc*I, respectively, have been used for typing *L. monocytogenes* with these methods. The discriminatory power of ribotyping was lower (DI = 0.873) than that of PFGE (DI = 0.946), as reported previously for *Eco*RI or *Pvu*II with manual ribotyping, and for *Apa*I or *Sma*I with PFGE [16,22]. However, this was substantially higher than the DI of conventional

Table 2. Subtypes of *Listeria monocytogenes* isolates defined by analysis of serotypes, ribotypes and PFGE types

Serotype	Ribotype	PFGE type	Final subtype	No. of isolates (n = 188)	Isolated from	
					Patients (n = 116)	Food-processing industry (n = 72)
1/2a	RT-1	5	1	17	14	3
1/2a	RT-1	44	2	1	1	0
1/2a	RT-1	66	3	1	1	0
1/2a	RT-1	V2	4	2	0	2
1/2a	RT-1	V9	5	1	0	1
1/2a	RT-1	V10	6	1	0	1
1/2a	RT-1	V13	7	1	0	1
1/2a	RT-1	V20	8	1	0	1
1/2a	RT-2	1	9	22	18	4
1/2a	RT-2	23	10	4	4	0
1/2a	RT-2	57	11	2	1	1
1/2a	RT-2	58	12	1	1	0
1/2a	RT-2	63	13	2	2	0
1/2a	RT-2	200	14	9	5	4
1/2a	RT-2	225	15	1	1	0
1/2a	RT-3	2	16	6	1	5
1/2a	RT-3	33	17	2	2	0
1/2a	RT-3	42	18	3	1	2
1/2a	RT-4	42	19	2	0	2
1/2a	RT-4	V1	20	2	0	2
1/2a	RT-4	V21	21	5	0	5
1/2a	RT-6	27	22	1	1	0
1/2a	RT-6	62	23	1	1	0
1/2a	RT-6	96	24	7	2	5
1/2a	RT-6	V4	25	5	0	5
1/2a	RT-8	53	26	1	1	0
1/2a	RT-8	V12	27	1	0	1
1/2a	RT-8	V15	28	1	0	1
1/2a	RT-8	V19	29	2	0	2
1/2a	RT-9	V16	30	1	0	1
1/2a	RT-9	V17	31	1	0	1
1/2a	RT-12	V3	32	2	0	2
1/2a	RT-13	V19	33	1	0	1
1/2a	RT-14	27	34	1	0	1
1/2a	RT-18	14	35	1	1	0
1/2a	RT-20	V7	36	3	0	3
1/2a	RT-23	202	37	1	1	0
1/2b	RT-21	64	38	1	1	0
1/2c	RT-1	V5	39	2	0	2
1/2c	RT-1	V6	40	2	0	2
1/2c	RT-1	V11	41	1	0	1
3a	RT-4	71	42	29	28[a]	1
3a	RT-10	V8	43	2	0	2
3a	RT-11	V18	44	1	0	1
3a	RT-22	244	45	1	1	0
3b	RT-7	V14	46	1	0	1
4b	RT-5	7	47	7	4	3
4b	RT-5	11	48	5	3	2
4b	RT-5	68	49	1	1	0
4b	RT-15	65	50	3	3	0
4b	RT-16	69	51	1	1	0
4b	RT-16	72	52	2	2	0
4b	RT-16	201	53	1	1	0
4b	RT-17	56	54	1	1	0
4b	RT-17	67	55	1	1	0
4b	RT-17	70	56	3	3	0
4b	RT-19	61	57	7	7	0

[a]At least 25 isolates were connected to an outbreak in 1998–1999 [12].

O:H serotyping (DI = 0.562). Although ribotyping was not as discriminatory as PFGE, it was rapid, simple to conduct, highly standardised, objective and labour-saving. The automated system was able to handle eight samples in 8 h, and four runs can be loaded during each working day. However, it is very expensive to purchase, the running costs are high, a good maintenance service is needed, and effective use of the system requires a comprehensive data base for the relevant strains. In contrast PFGE is a time-consuming method, although a 30-h standardised protocol has been described for *L. monocytogenes* [35] which has made it more competitive with automated ribotyping.

Table 3. Number of ribotypes and PFGE types in each serotype

Serotype	No. of isolates	No. of ribotypes	No. of PFGE types
1/2a	116	13	34
1/2b	1	1	1
1/2c	5	1	3
3a	33	4	4
3b	1	1	1
4b	32	5	11

Table 4. Number of serotypes and PFGE types in each ribotype

Ribotype	No. of isolates	No. of serotypes	No. of PFGE types
RT-1	30	2	11
RT-2	41	1	7
RT-3	11	1	3
RT-4	38	2	4
RT-5	13	1	3
RT-6	14	1	4
RT-7	1	1	1
RT-8	5	1	4
RT-9	2	1	2
RT-10	2	1	1
RT-11	1	1	1
RT-12	2	1	1
RT-13	1	1	1
RT-14	1	1	1
RT-15	3	1	1
RT-16	4	1	3
RT-17	5	1	3
RT-18	1	1	1
RT-19	7	1	1
RT-20	3	1	1
RT-21	1	1	1
RT-22	1	1	1
RT-23	1	1	1

Although the benefit of serotyping is limited, it is a rapid screening method if an outbreak is suspected [12]. In the present study, the most common serotypes were 1/2a (62% of isolates) and 4b (17%). The high incidence of isolates belonging to serotype 3a (18%) is a result of the link with an outbreak in 1999 [12]. Isolates with this serotype were also identified from 1997 and 1998, although, at that time, the outbreak was not detected. However, in 1998, three food isolates of this rare serotype were isolated from food sources other than butter, and had different ribotypes and PFGE patterns from those causing the outbreak.

Only five (7%) isolates of serotype 4b were isolated from food industry samples, whereas 27 (23%) human isolates belonged to this serotype. This supports earlier findings that, even though serotype 4b has caused a number of outbreaks, it has been isolated rarely from foodstuffs, which are contaminated mainly by serotype 1/2 isolates [36,37]. Buncic *et al.* [38] found that 1/2a isolates tended to be more resistant than 4b isolates to two tested anti-listerial bacteriocins at 4°C. However, after cold storage at 4°C, 4b isolates tended to be more resistant to heat treatment at 60°C, and were more pathogenic than 1/2a isolates when transferred from cold storage to body temperature. These findings may explain why 1/2a isolates are obtained more often from foodstuffs in industrial processing, while 4b has caused more outbreaks.

In the present study, only 18% of the final subtypes included both human and food industry isolates, although the isolates were collected during the same time period. However, two final subtypes (1 and 9) predominated among human isolates, and identical subtypes were also obtained from food-processing plants in Finland. In addition, 26% of PFGE types identified among food industry isolates had never been found among human isolates from Finland in 1990–2001. It would be interesting to compare the pathogenicity of isolates representing PFGE types isolated frequently from invasive infections and that of isolates not identified among human pathogens.

PFGE patterns were serotype-specific. However, three PFGE types divided into two different ribotypes each and, in three cases, ribotyping was more discriminatory than PFGE. In general, one ribotype included isolates of only one serotype, except RT-1 and RT-4, which included isolates belonging to two different serotypes, namely 1/2a and 1/2c, and 1/2a and 3a, respectively. This is in agreement with previous results [15]. In addition, the ribotype profiles of the isolates within these serotypes have been reported to belong to the same genetic ribotype subgroup [15,20].

In the present study, the isolates of one ribotype belonged to several PFGE types; for example, RT-1 isolates had 11 PFGE types, while RT-2 isolates had seven PFGE types, and RT-4 isolates had four PFGE types, with similarity values of 50–93%, 75–100% and 70–87%, respectively. Isolates with PFGE profiles differing by more than seven fragments are unrelated according to Tenover *et al.* [39]. This corresponds to similarity values of *c.* 70% or less, indicating that some of the RT-1 and RT-4 isolates are unrelated. Since any difference between two profiles was considered sufficient to distinguish two PFGE profiles, some of the isolates with these PFGE types might also have been related closely. In large ribogroups, which include patterns of many different isolates in the database, the ability to accept a new pattern is lower than in small groups including only patterns

of one isolate. For example, ribogroup RT-1 already contains 286 patterns with a mean similarity value of 0.98 ± 0.03. This suggests that automated ribotyping has limits in its applications, especially for closely related isolates and large ribogroups.

An international collaborative study sponsored by the World Health Organisation reported that the discriminatory power of manual ribotyping with *Eco*RI for serotype 4b may not be adequate for epidemiological investigations [21]. In the present study, the ribotyping DI was lower for serotype 4b (0.762) than for the most common serotype 1/2a (0.803). However, De Cesare *et al.* [40] studied the suitability of 15 different enzymes for discrimination of *L. monocytogenes* isolates in automated ribotyping and found that the use of other enzymes (*Pvu*II, *Eco*RI, *Xho*I) may improve discrimination significantly among isolates of serotype 4b. As most of the serotype 3a isolates originated from an outbreak, a low DI value was recorded.

RT-4 included nine isolates of serotype 1/2a originating from food industry samples with three PFGE types, and 29 isolates of serotype 3a representing PFGE type 71, including the isolates connected to the outbreak in Finland in 1999 [12]. According to these results, ribotyping with *Eco*RI alone was not sufficient to discriminate the outbreak isolates of serotype 3a. Although the benefit of serotyping is usually considered to be limited, it was useful in this outbreak caused by isolates with a rare serotype. Thus, ribotyping and serotyping were a useful combination, and serotyping alone, despite its low DI, may reveal the emergence of an epidemic subtype, as it did in Finland in 1999 [12]. Automated ribotyping is a useful tool in the first stages of an epidemiological survey, as a large number of isolates can be analysed rapidly and it is not labour-intensive. However, during an outbreak, suspected outbreak-associated isolates must be investigated by a more discriminative method, as is commonly recommended for other subtyping methods [16,41,42], but use of another restriction enzyme might increase the discrimination provided by automated ribotyping [40,43].

ACKNOWLEDGEMENTS

This work was supported in part by the National Technology Agency (TEKES), the Finnish Research Programme on Environmental Health. The skilful technical assistance of H. Hakuli, T. Heiskanen, L. Immonen, U.-M. Nakari and R. Taipalinen, the editorial assistance of M. Koukkari, and the critical reading of the manuscript by M. Saarela, are gratefully acknowledged.

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